THE EFFECT OF REPEATED BLEEDINGS ON THE BLOOD CONSTITUENTS OF IMMUNISED HORSES.

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(*From the Wellcome Physiological Research Laboratories.*)

(With 3 Charts.)

One of the phenomena in immunity not completely explained is the persistence of demonstrable antibody in patients for many years after recovery from an acute infection. A typical instance is that of a person maintaining a high agglutinin titre for years after an acute typhoid infection.

One obvious explanation that might suggest itself is that all such persons are "carriers" and that they are constantly being reimmunised by small doses of bacterial poison from the typhoid bacilli in gall bladder, intestine, or elsewhere. It is easy to extend such an hypothesis and to imagine that all immunity after natural infection is due to the persistence of the living agent of the infection in some concealed site in the body and the resultant frequent immunisation of the patient by the foreign protein of the infecting agent. But leaving such debatable ground and restricting the discussion to immunisation produced by non-living protein, we start with several facts.

A rabbit which has been passively immunised with rabbit or other serum containing antibody will lose practically the whole antibody from the blood in a few weeks or months (Jörgensen and Madsen, 1902).

This leads one to expect that *destruction or elimination* of antibody is constantly occurring also in the actively immunised animal. Since the titre in the latter case falls more slowly than does that of a passively immunised animal, one has to assume that antibody is constantly

appearing in the blood for a very long period after active immunisation to replace that destroyed, in other words, that antibody is being produced long after the probable disappearance of the antigen.

It has often been stated that antibody may be formed in response to the injection of non-specific antigen (Dreyer and Walker, 1909). An injection of dead staphylococci for instance is said to cause an increase in the titre of typhoid agglutinins in an animal immunised with *B. typhosus* and it is also stated that such non-specific influences as bleeding can cause the production of antibody (Roux and Vaillard, 1893). But as far as I can find, in all the experiments reported, *recently* immunised animals were dealt with so that circulating antigen was still possibly present and still able to exert its influence[1]. Further, what was ascertained was the *titre* and not the total amount of antibody in the whole blood or in the whole of the tissues. Without these data it seems somewhat unsafe to conclude that antibody is actually produced anew.

Thus three procedures are really necessary before one can say that antibody has been produced as a result of bleeding, viz.

1. Estimation of titre,
2. „ „ total blood—and from these two factors, total antibody in the blood.
3. „ „ antibody in the tissues.

It may be advisable to refer briefly to certain experimental work on this question and to the conclusions reached.

Summary of Previous Work on the Influence of Bleedings on Antibody-content.

Roux and Vaillard (1893) immunised two rabbits against tetanus toxin, ascertained the antitoxin-titre and then from one of them took 200 grms. of blood in the course of 20 days. They found that its titre two days after the bleeding had fallen only as much as that of the control immunised rabbit which had not been bled.

Salomonsen and Madsen (1898) immunised a mare against diphtheria and very shortly afterwards drew off seven litres of blood and found a drop in the antitoxic titre of the animal's blood and milk of about

[1] The theory that residual antigen gave rise to this apparently new production of antibody after bleeding was suggested by Rothberger (1906) though no definite experimental evidence was brought forward in its support.

one-seventh. They bled a second horse soon after injection, when the curve of antitoxin titre was mounting and noted a drop of 35 %, but after a period of 12 days the titre was again at the previous level. In the course of seven days they took from a goat immunised with diphtheria toxin eleven-twelfths of its blood-volume and found a drop to 17 % of the initial titre, but six days later it had reached 62 % of the initial titre. Their conclusion was that the figures indicated a new production of antitoxin and that certain cells in the organism had acquired a new and persistent secretory power.

Nicolle (1904), working with rabbits, showed that the descent of the curve representing the titre of agglutinin for typhoid bacilli could be temporarily arrested by bleeding. Friedberger and Dorner (1905), in the case of rabbits immunised with goat corpuscles, bled the animals 10 to 20 c.c. either before or just after injection and found the titre in the bled animals was about four times that of the non-bled. In view of the therapeutic use of bleeding in the treatment of many diseases it is of interest to note that the favourable influence exerted by bleeding in these experiments was most marked where the bleeding was done two to three days after the injection.

Lüdke (1904) reported some results of bleeding in rabbits immunised with ox blood. He took very large amounts of blood, such as 80 to 100 c.c., from the rabbits at successive bleedings and apparently found that the titre became raised some days after the bleedings, but as he speaks of such quantities as "three to six drops of the serum" as being necessary to dissolve 1 c.c. of 4 % ox blood corpuscles, one cannot draw very clear deductions from his work.

The same author (Lüdke, 1906) took 80 c.c. of blood in five days from a rabbit previously injected with ox blood, and one day later found the haemolytic titre unaltered. (Tables and full details were not given.)

Schroeder (1909), who reviewed the whole literature of the subject, described many experiments on rabbits immunised with *B. coli communis* and *B. typhosus*. He applied Madsen's equation for calculating any point on the curve of agglutinin development after a single injection. He then bled his rabbits and subsequently ascertained their titre and noted whether the point fell (*a*) on, (*b*) above, or (*c*) below the calculated point. His conclusion was that the bleeding had (*a*) left unaltered, (*b*) increased, (*c*) decreased the agglutinin content.

Bleeding was performed usually during the stage of falling curve and he found that the fall was delayed or even replaced by a temporary rise.

He therefore concluded that after such bleeding a considerable reproduction of agglutinin takes place. He apparently did no blood-volume determinations and did not investigate the distribution of agglutinin throughout the various tissues.

Dreyer and Walker (1910) stated that by repeated bleedings one can keep up the percentage of antibodies in the blood long after it would have fallen to a low level and can even cause an increase in the antibodies above the former maximum.

As a result of these various experiments by various workers clinicians have concluded that bleeding causes a rise in the agglutinin-titre and, making the natural assumption that a rise in agglutinin-titre is to the advantage of the patients, have taken from typhoid fever patients one or two hundred c.c. of blood in order to raise the agglutinin titre. This has been done by various physicians in Germany and by Schroeder in Denmark and has been recommended by Whitehead in England (1911).

Object of present experiments.

The experiments here brought forward deal with results observed after numerous bleedings. They were carried out on horses, with which animals estimation of total blood-volume or of the total amount of antibody in the tissues is not an easy matter, and also on rabbits, in which these estimations can be made.

Description of Experiments on the Horse.

Methods. 225 c.c. of a 7·5 % suspension of sheep's red cells were injected into a horse which rapidly attained a high haemolytic titre. Two months later the haemolytic titre was found to be at a constant level over a period of three to four weeks. Various quantities of blood were then withdrawn at intervals of a week or more with occasional resting periods, so that in 11 months a total quantity of 122 litres was taken from the jugular vein.

Titration. After many trials with suspensions of red cells of varying strengths, readings after varying periods, etc., 0·5 c.c. of a 1 % suspension of the centrifugalised deposit of red cells, which deposit contained about 26 to 28 million cells per c.c., was mixed with 0·05 c.c. of fresh guinea-pig serum, 0·5 c.c. of the dilution of the horse's serum to be tested, and made up to 2·5 c.c. with 0·9 % saline solution. The tubes were finally placed in a water-bath at 37° C. and the decisive reading was that taken

after one hour. It was not difficult to find the end point when each successive tube contained 20 % less than the preceding one. When it contained 10 % less, care had to be taken to have the surfaces of the tubes carefully freed from traces of dirt, but in bright daylight and with clean water in the bath it was generally possible to make a clear distinction between successive tubes.

The guinea-pig complement used was titrated each time with a fixed dose of red cells and of old stable haemolysin. The extreme limits of variation were between 0·01 c.c. and 0·014 c.c., but very rarely was the reading outside ·01 c.c. to ·012 c.c.

The individual specimens were tested as soon as possible after the bleeding and periodically batches of three, six or eight samples were titrated against each other. For the later bleedings an old stable haemolysin, obtained from another horse, was always used as a "standard," a procedure similar to the German method of estimating tetanus antitoxin.

In Table II the values of the haemolytic titre are recorded. Occasionally they will be seen to vary fairly widely, but where a wide discrepancy was observed numerous subsequent titrations were made of the same specimen, compared with previous bleedings and the "standard," and the average of the several readings is the point shown in Charts 1 and 2.

Red and white cells were enumerated with a Bürker haemacytometer, two duplicate samples of 1 c.c. of blood being usually counted. (Average error about 10 %.) *Haemoglobin* was estimated in duplicate or triplicate with a Gowers instrument—human scale. (Average error about 3 %.) For *specific gravity* two bottles, each containing 10 c.c. of blood, were weighed. The blood was caught from a cannula in the vein and immediately filled into the bottles. These were kept at laboratory temperature, which varied from about 10° C. to 20° C. From Table II it will be seen that the extreme difference between the duplicate readings was two in the fourth figure. Taking the last two figures of readings of the samples to represent comparative percentages this gives an average error of about 4 %. For *differential counts* usually about 400 white cells were counted, checked at important points by two such counts. (Average error about 10 %.)

The total proteins were estimated by refractometer readings checked and interpreted by coagulating the total proteins and weighing the precipitate at many points. (Average error about 2 %.)

TABLE II.

Effect of repeated bleedings on constituents of blood of immunised horse.

Horse 1.

Date	Amount bled litres	Red cells per c.mm.	Haemo-globin	Specific gravity	Total protein	Leuco-cytes	Differential count: total counted	Differential count: polymorpho-nuclear cells (%)	Differential count: mono-nuclear cells (%)	Differential count: eosinophile (%)	Differential count: mast cells (%)	Haemo-lytic titre
6. 2. 12	4	10,200,000	95·1	1048	6·45	9100	169	59	35	7		·0016
		100	**100**	**100**	**100**	**100**						
10. 2. 12		9,600,000	83			9400						··0016
		94	**87**			8550						
						100						
15. 2. 12		9,040,000		1047	6·45	8650						
		89		**97**	**100**	8800						
20. 2. 12	4			1047	6·5	**98**						
				97	**100·5**							
22. 2. 12		10,200,000		1046	6·31	11400						·0016
		100		**95**	**98**	10200						
						118						
1. 3. 12	4	8,650,000	80	1046	6·32	7250	472	60	34	6		
		8,300,000	**84**	**95**	**98**	**81**						
		84										
8. 3. 12	4	8,160,000		1046	5·81	8150						·0030 (?)
		80		**95**	**90**	**91**						·0016
												·0016
15. 3. 12		7,680,000	83	1049	6·39	10450	576	69	26	4	1	·0016
		75	**87**	**103**	**99**	**117**						
20. 3. 12	9	9,660,000			6·41	7200						·0016
		94			**99·5**	**79**						
22. 3. 12		8,460,000		1046		10000	314	70	27	3	1	·0016
		8,620,000		**95**		10350						·002
		84				**113**						
25. 3. 12							308	65	30	5		·0008 (?)
												·0016
												·0016
2. 4. 12		9,780,000	90	1055	6·91	7600	441	68	26	5		·001
		95	**95**	1054	**112**	**86**						
				113								
17. 5. 12	4	10,400,000		1059		11000	437	68	23	8	1	·0016
		9,930,000		1059		**124**						
		100		**122**								
23. 5. 12	4	9,920,000		1052		7900						·0016
		97		1052		**89**						
				108								

Haemolytic titre = the amount in c.c. of serum necessary to completely dissolve ·5 c.c. of 5 % red cell suspension with ·05 c.c. fresh guinea-pig serum in one hour at 37° C.

The figures in heavy type represent percentages.

Date	Amount bled litres	Red cells per c.mm.	Haemo-globin	Specific gravity	Total protein	Leuco-cytes	Differential count: total counted	Differential count: polymorpho-nuclear cells (%)	Differential count: mono-nuclear cells (%)	Differential count: eosinophile (%)	Differential count: mast cells (%)	Haemo lytic titre
29. 5. 12	4	9,408,000		1041		7200	392	76	19	4		·0016
		8,605,000		1041		**81**						
		88		**85**								
3. 6. 12	4	8,000,000		1046		7300	378	71	26	3	1	·0016
		79		**95**		**82**						
11. 6. 12	6	9,970,000	84	1044		13200						
		97	**86**	1045		**149**						
				91								
17. 6. 12	6	8,320,000	70	1048		6400	446	67	31	2		
		8,080,000	**73**	**100**		6900						
		81				**74**						
24. 6. 12	6	7,500,000		1044	6 21	8000	442	68	28	3	1	·0016
		7,280,000		1045	**96·5**	8600						·0016
		73		1044		**83**						
				91								
2. 7. 12	6	7,080,000	84	1046	6·35	6660	423	71	25	3	1	·002
		6,640,000	**88**	1046	**98**	6700						·0016
		67		1044		**74**						
				92								
2. 9. 12	5	9,520,000	90	1052		4400						·002
		8,880,000	**95**	1054		5300						
		89		**110**		**56**						
10. 9. 12	6											·002
												·0016
												·0024
27. 9. 12	6	7,960,000	76	1043	6·35	5600	350	69	23	7	1	·002
		8,320,000	**80**	1045	**98**	4800						·002
		80		**92**		**60**						
16. 10. 12	6				6·61							
					102							
25. 10. 12	6				6·48							
					100·5							
2. 11. 12	6				6·21							·0024
					96							
8. 11. 12	6				6·11							
					94·5							
16. 11. 12	6	6,770,000	75	1044	6·2	6660	430	70	22	7	1	·0024
		8,000,000	**79**	**91**	**96**	6400						
		72				**73**						
19. 12. 12	10	8,800,000	104	1057	7·2	6000						·0024
		87	**109**	**118**	**111**	**68**						
31. 12. 12		6,320,000	74	1047	6·7	8600						
		6,160,000	**73**	**97**	**104**	8500						
		61				**92**						
3. 2. 13		8,160,000	93	1053	6·8	6000						
		80	95	**110**	**105**	**68**						
			100									

Results.

When one considers the results as a whole, the small effect that the bleedings had on the animal is particularly striking. Table II gives details of the effects of the bleedings. In Charts 1 and 2 the amounts of each blood constituent are calculated as percentages of the amounts present at the beginning of the experiment; Chart 1 shows the variations of haemolysin, red cells and haemoglobin; Chart 2 those of haemolysin, specific gravity, total protein and leucocytes throughout the

Constituents of the blood expressed as percentages of amount present at beginning of the experiment

Chart 1. Effects of repeated bleedings on haemolysin, haemoglobin and red cells of an immunised horse. Period covered by chart = 11 months. Total bleeding = 120 litres.

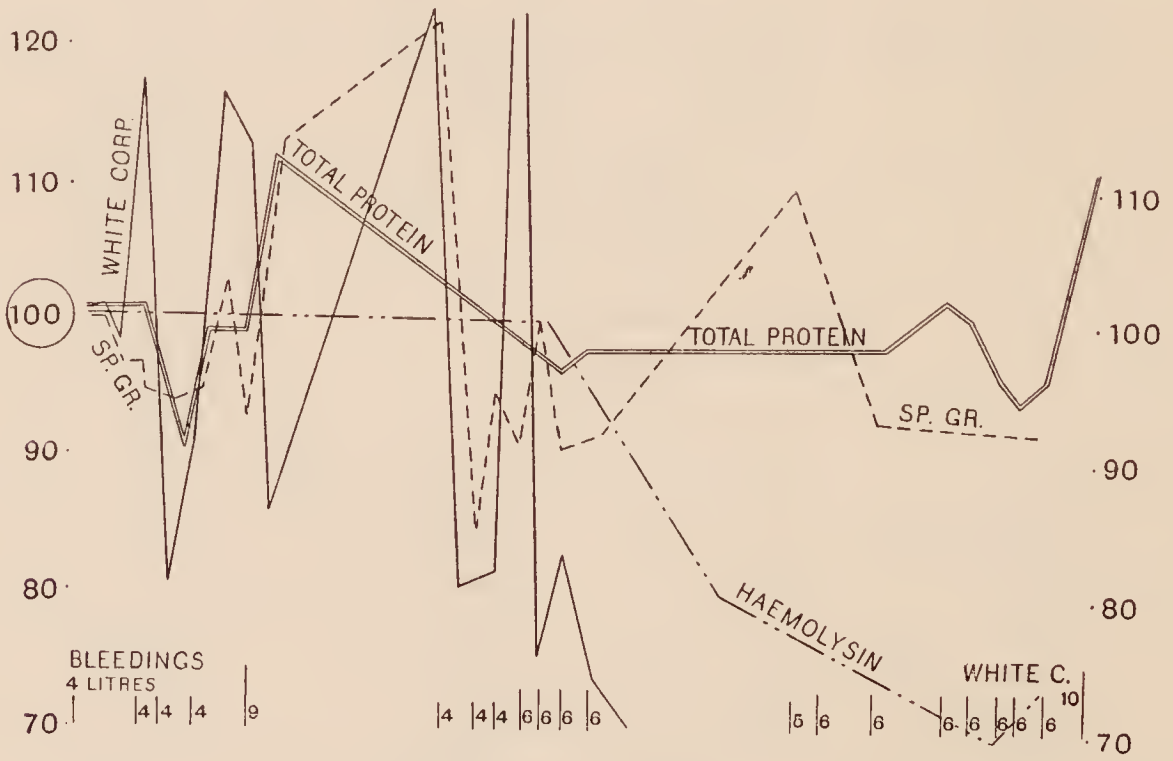

Chart 2. Effects of repeated bleedings on haemolysin, leucocytes, specific gravity and total protein of an immunised horse. Period covered = 11 months. Total bleeding = 120 litres.

experiment. *Haemolytic titre* was but slightly affected[1]. During the year (1912) the titre has gradually dropped to 66 % of the original, the fall commencing in June, by which time 40 litres had been withdrawn, and being accelerated by the September, October and November bleedings which amounted to 58 litres.

Other constituents. The red cells and haemoglobin (the colour index varying very little from unity) and the specific gravity showed considerable fluctuations and at the end of the experiment are at a somewhat lower level that at the commencement. The power of maintaining the protein content is very striking and the horse at the end of 12 months has a higher protein content than at the beginning of the bleedings.

The rapid rise in all constituents during the resting periods April–May and July–August shows how active the power of repair remained. This ability to undergo repeated large bleedings of eight litres each with but slight subsequent fall in blood constituents is also seen in Table III and Chart 3 taken from another horse under observation (horse 2).

TABLE III.

General results of repeated bleedings amounting to 197 *litres = four to five times total blood-volume, in a period of* 11 *months.*

Horse 2.

	Specific gravity	Red cells per c.c.	White cells per c.c.	Differential count: total counted	poly-morpho-nuclear cells (%)	mono-nuclear cells (%)	eosino-phile (%)	mast cells (%)	Haemo-globin	Total protein (%)
25. 3. 12	1055	9,000,000	8800	334	60	34	6	0	74	8·0
Total bleedings 64 litres	**100**	**100**	**100**						**100**	**100**
3. 7. 12	1039	6,288,000	8800	422	71	24	4	0	70	7·2
Total bleedings 48 litres	**71**	**70**	**100**						**95**	**90**
27. 9. 12	1044	6,840,000	6,530						70	7·5
Total bleedings 36 litres	**80**	**76**	**85**						**95**	**93**
18. 11. 12	1042	6,800,000	10000	511	59	35	5	1	64	6·3
Total bleedings 49 litres	**76**	**76**	**115**						**86**	**79**
18. 2. 13	1045	6,400,000	8000						65·6	6·8
	82	**70**	**91**						**88**	**85**

The figures in heavy type represent percentages.

[1] From results published elsewhere (*Journ. of Path. and Bact.* xviii. 90) it appears that this horse can keep its haemolytic titre curiously constant even after a 10 litre bleeding.

The relation between Antibody Curve and that of other constituents of the blood.

To revert to a consideration of the results obtained with horse 1 it was hoped that a study of the curves would reveal a close association between one or other of the various blood constituents and the antibody and so perhaps suggest a site for the manufacture of antibody—but no such association appears. The only constituent that departs widely from the haemolysin curve and that of the other constituents is the white cell content, which fact is of interest, when one recalls the view so often advanced that the leucocytes or the tissues where they originate are the main sites of antibody manufacture.

The one interesting fact that emerges is that although in a series of bleedings commenced three months after the last injection of sheep's red cells, 122 litres of blood were taken from the horse its haemolytic

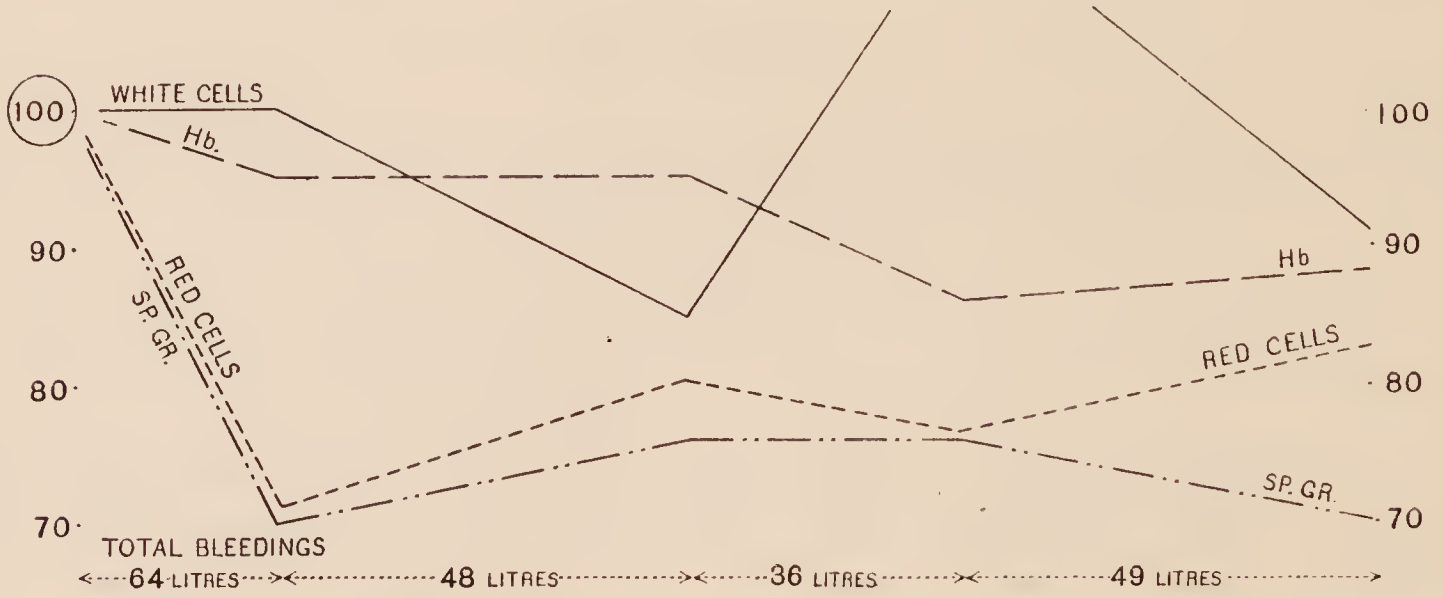

Chart 3. Effect of repeated bleedings on blood of horse 2. Constituents expressed as percentages of figures at start of experiment. A total of 197 litres withdrawn in 11 months.

titre is still 66 % of the constant titre attained before the bleedings were commenced. This amount of blood probably represents $2\frac{1}{2}$ to $3\frac{1}{2}$ times the total blood-volume. This assumption is founded on the following experimental data:

(A) In a series of some 30 horses bled out from the carotid artery the average amount of blood obtained was 27 litres. From a rabbit or dog one can bleed out $\frac{3}{4}$ of its total blood-volume. The use of this factor of $\frac{3}{4}$ would make the total blood-volume of the average horse about 36 litres.

(B) One can inject 5 to 10 litres of saline into one jugular vein of a horse in about 8 to 14 minutes, and immediately afterwards take a sample from the opposite jugular and estimate the fall in the content of red cells, haemoglobin, total proteins or specific gravity. By treating the experiment as a dilution *in vitro* and by making no allowance for the unknown rate at which absorption and elimination immediately proceed, the blood-volume may be calculated. This procedure has given me figures like 42, 45, 51 and 53 litres. I am endeavouring at present by Schürer's modification of this injection method to obtain better figures than these, but meanwhile assume that the average horse contains 35 to 45 litres of blood.

It is certain that the initial figure would have dropped somewhat during the year, apart from any interference, so that we can safely conclude

(1) That the series of bleedings has had only a limited adverse influence on the titre.

(2) That the horse has either produced afresh something like three times the amount of antibody in the blood stream at the commencement of the bleedings or that this amount of antibody may have come from some pre-existing store of antibody in the tissues.

The Question of a Reservoir of ready made antibody in the tissues or lymph.

This question was investigated on rabbits immunised long previously with sheep's red cells and therefore possessing a fairly constant titre. Four experiments were performed. In one (*v.* Table IV) the rabbit had not been previously bled more than a few c.c. at a time; the other three had been subjected to one or two bleedings of about 20 c.c. each, during the week preceding the experiment. Each of these preliminary bleedings was followed by such a production or a readjustment of distribution of antibody that the haemolytic titre remained practically constant and it was hoped that if the faculty of storing in the tissues ready made haemolysin existed, such faculty would be enhanced in response to the bleeding and so render the storehouse more easily detected.

Technique. The rabbit was bled out from the carotid and then about $2\frac{1}{2}$ litres of Ringer's fluid were run through from the aorta. The washings, obtained from the right auricle, were caught in successive jars in each of which the red cells and antibody were estimated; the spleen, liver, appendix, kidneys, brain and samples of muscle and marrow

were ground up separately and extracted with saline solution. Each extract was tested for antibody.

The results are given in Tables IV, V, VI and VII. Making allowance for the difficulty of estimating haemolysin accurately in these washings, it seems fair to conclude that there is no great store of ready made detectable haemolysin that can be washed out or extracted by grinding and washing[1].

It was conceivable that haemolysin was present in these organ extracts but not detected because of some inhibitory principle also

TABLE IV.

Haemolytic rabbit bled out, then washed out with 2 litres of Ringer's fluid.

	Diluted blood c.c.	Percentage of red corpuscles	Volume of blood c.c.	Percentage of total blood	Percentage of total haemolysin
Jar 1	—	100	45	60	75
Ringer commenced					
Jar 2	250	7·2	18	24	13
,, 3	750	1·6	21	14	8
,, 4	250	·51	1	1	2
,, 5	500	·1			1
		organs and limbs massaged			
,, 6	250	·2			·3
,, 7	100	·3			·05

TABLE V.

Haemolytic rabbit (bled 40 c.c. during previous week) bled out, washed out with $2\frac{1}{2}$ litres of Ringer's fluid, organs ground up and haemolysin content determined.

	Diluted blood c.c.	Percentage of red corpuscles	Volume of blood c.c.	Percentage of total blood	Percentage of total haemolysin
Jar 1	—	100	65	72	44
,, 2	150	7·5	10	11	10
,, 3	540	1·86	10	11	9
,, 4	1280	·32	4	4	21
,, 5	540	·25	1	1	12
Muscle, appendix, brain, spleen, liver, kidneys together contained about					4

[1] Dreyer and Ray (*Journ. Path.* XIII. p. 344, 1909) state that they were able to wash out of the tissues 30 % of the total agglutinins obtained. These results apparently do not run parallel with those obtained from rabbits long previously immunised with red cells.

TABLE VI.

Rabbit (bled 40 c.c. during previous week) bled out, washed out with 2½ litres of Ringer's fluid, organs ground up and haemolysin content determined.

	Diluted blood c.c.	Percentage of red corpuscles	Volume of blood c.c.	Percentage of total blood	Percentage of total haemolysin
Jar 1	—	100	54	79	98·2
,, 2	260	1·4	3	4	1
,, 3	1000	·82	8	12	·6
,, 4	1300	·26	3	4	·1
Appendix, brain, spleen, liver, kidney and muscle together contained about					1

TABLE VII.

Rabbit (bled 25 c.c. two days previously) bled out, washed out with 4 litres of Ringer's fluid, organs ground up and haemolysin content determined.

	Diluted blood c.c.	Percentage of red corpuscles	Volume of blood c.c.	Percentage of total blood	Percentage of total haemolysin
Jar 1	—	100	70	76	61
,, 2	500	2	10	11	8·6
,, 3	500	1·1	5	5	6·5
,, 4	1000	·3	3	3	10·6
,, 5	500	·1	·5	·5	·8
,, 6	1425	·3	4	4	12·4
Liver, spleen, kidney, muscles and marrow together contained about					·8

present in the extract. To test this a normal rabbit's tissues were ground up and a certain volume of the paste of various organs mixed with an equal volume of rabbit serum containing a known quantity of anti-sheep haemolysin and allowed to remain in contact for some hours. The haemolysin in the supernatant fluid was again estimated, but no evidence was found of any marked inhibitory influence in pastes of spleen or muscle and only slight inhibition was caused by liver paste.

Summary of Results.

(1) From a horse injected with sheep's red cells three months prior to the first bleeding and in a condition of constant haemolytic titre 122 litres of blood were taken in a period of 11 months. The horse's condition remained good throughout and has markedly improved during the year.

(2) The net result was that the haemolytic titre during that time fell only to about 66 % of its original value, the leucocytes to about 66 %, haemoglobin scarcely at all, while the specific gravity of the blood and total protein have increased, the former by 10 %, the latter by 5 %.

(3) There was no relationship between the total number of leucocytes[1] and the amount of antibody. The differential count showed an increase of 12 % in the polymorphonuclear and a decrease of 12 % in the mononuclear cells, these figures being not very far outside the experimental error. The eosinophile and mast cells showed no marked alteration in number, size or staining reactions.

Discussion of Results—Conclusions.

(1) A horse can be trained to withstand without any recognisable ill effect on its general health, and with remarkably little diminution in blood constituents, very large bleedings, amounting to several times its total blood-volume in less than a year. By allowing a considerable time to elapse after injection, it was expected that one would get further away from the effect of the presence of antigen and so hope to obtain a clear view of the effect of bleedings.

Whether a foreign protein may resist destruction in the body for months we do not know. But it is not probable, for if one injects by any parenteral method a serum containing an antibody, *e.g.* diphtheria antitoxin, it is found that in a very short period such antibody is no longer detectable and has presumably been destroyed. We know further that when one injects an easily recognisable foreign protein such as nucleated bird corpuscles into a mammal, the nucleated foreign cells rapidly disappear from the blood stream and undergo phagocytosis in the haemopoietic tissues.

It is therefore probable that the sheep cells injected in October 1911 had been eliminated or destroyed before February 1st, 1912, and that any subsequent alterations of haemolytic titre were not dependent on residual antigen. Experiments on rabbits (*v.* Table I) showed, firstly, that antibody (haemolysin) may remain nearly constant in titre after bleeding though the blood-volume (Boycott, 1909) increases, and, secondly (*v.* Tables IV, V, VI, VII), that the amount of ready made haemolysin in the tissues is negligible.

[1] It was not possible to take samples at the same hour every day. They were taken between 10 a.m. and 5 p.m. but mostly about 3 p.m.

TABLE I.

Small alterations of haemolytic titre following bleeding rabbit 1, bled 20 c.c. on Sept. 9, 1912.

Technique. Guinea-pig serum 0·05 c.c., 1 % red cell emulsion 0·5 c.c., saline added to 2·5 c.c., water-bath at 37°, reading after one hour.

Quantity of serum	Sept. 9, 1912	Sept. 10	Sept. 11	Sept. 12	Sept. 13	Sept. 14
0·0016 c.c.	‡	‡	‡	‡	‡	‡
0·00128	‡	‡	‡	‡	‡	‡
0·00102	‡	‡	‡	‡	‡	‡
0·0008	‡	‡	‡	+	+	+
0·0006	+	+	+	±	±	+

‡ = complete haemolysis.
‡ ‡ ‡ ± indicate grades of haemolysis.
+ = very slight haemolysis.

This table is typical of five such experiments, the amounts bled being from 15 c.c. to 35 c.c.

We may assume then that there was no storehouse or reservoir of formed haemolysin in the tissues on which the horse could have drawn during the eleven months of the experiment, and since $2\frac{1}{2}$ to $3\frac{1}{2}$ times its total blood-volume was withdrawn, one may consider the conclusion definitely proved that

(2) The cells of an immunised animal have acquired a persistent faculty of manufacturing haemolysin and can do so in response to (or possibly in spite of) repeated extensive bleedings.

With regard to the site of production of antibody, it is difficult to correlate the haemolysin production with variations in the quantitative proportions of cells of the haemopoietic system or of the protein constituents of the serum.

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